American Journal of Hematology 62:82–87 (1999)

Minimal Evidence of Platelet and Endothelial Cell Reactive Antibodies in Thrombotic Thrombocytopenic Purpura

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Thrombotic thrombocytopenic purpura (TTP) is a syndrome characterized by microvascular thrombosis with thrombocytopenia and end-organ injury. Evidence suggests that platelet or endothelial cell injury may be initial pathological events in TTP. A number of factors in patient plasma, including immunoglobulins, have been proposed to mediate cellular injury in TTP. However, systematic analyses of TTP patient plasma for the presence of platelet or endothelial cell antibodies are lacking. We, therefore, analyzed 48 TTP patient plasma samples for the presence of platelet and endothelial cell antibodies by using enzyme-linked immunosorbent assay, flow cytometry, and microlymphocytotoxicity. Twelve of 48 TTP patient samples (25%) reacted against purified platelet glycoproteins. Nine (19%) also contained antibodies that bound to allogeneic target platelets in flow-cytometric assays. Nine of 48 samples (19%) contained antibodies to human umbilical vein endothelial cells in flow-cytometric assays, and seven of 48 patient samples (15%) bound to human dermal microvascular endothelial cells. Six of 48 (13%) patient plasma samples contained antibodies that bound to human umbilical vein endothelial cells activated with γ-interferon and tumor necrosis factor-α. Of twenty samples that were reactive in one or more platelet or endothelial cell assay, eight contained human leukocyte antigen antibodies reactive in microlymphocytotoxicity. These studies demonstrate that antibodies reactive against platelet or endothelial cell antigens are not prevalent in TTP, and that more than a third of antibodies detected are human leukocyte antigen alloantibodies. Our findings suggest that autoantibodies against platelets or endothelial cells are not important in the pathogenesis of this syndrome. Am. J. Hematol. 62:82–87, 1999. © 1999 Wiley-Liss, Inc.

Key words: thrombotic thrombocytopenic purpura; platelet; endothelial cell; antibody

INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a syndrome characterized by microvascular thrombosis associated with hematological abnormalities and end-organ damage [1]. Although the pathophysiology of microvascular thrombosis in TTP remains obscure, evidence indicates that activation and injury of platelets and endothelial cells are critical events [1,2]. A number of investigators have reported the presence of platelet or endothelial cell activating factors in the plasmas or sera of patients in the acute phase of TTP [3–6].

A possible role of humoral immunity in mediating microvascular injury in TTP has long been speculated. Similarities between TTP and autoimmune thrombocytopenic purpura have provided rationale for use of corticosteroids and splenectomy in TTP [7,8]. Associations between TTP and systemic lupus erythematosis (SLE), the antiphospholipid antibody syndrome, and other autoimmune disorders provide further cause for suspicion of immunological mechanisms [1,9,10].

The suspected presence of autoantibodies against platelets or endothelium in TTP has prompted exploration of a number of adjuvant treatments. In addition to

Contract grant sponsor: National Heart Lung and Blood Institute; Contract grant numbers: HL 55556 and HL 13629.

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Received for publication 15 April 1998; Accepted 2 June 1999

corticosteroids, immune modulating agents such as vincristine, cyclosporin, azathioprine, intravenous immunoglobulin, and staphylococcal protein A adsorption column therapy have been reported to be effective in some patients [11–16].

Because antibodies have been implicated in the etiology of TTP, and treatments involving immune modulation are often used to treat patients despite few data supporting a humoral immune mechanism, we conducted a systematic analysis of acute-phase TTP plasma samples for anti-platelet and anti-endothelial cell antibodies.

MATERIALS AND METHODS

Plasma was obtained from 48 patients treated in the acute phase of TTP following informed consent. Minimum diagnostic criteria included thrombocytopenia and microangiopathic hemolytic anemia with elevated lactate dehydrogenase and minimal or no evidence of coagulopathy. Plasma samples were obtained from ehtylediaminetetraacetic acid anticoagulated whole blood samples collected prior to initial plasmapheresis, or from the first 300 ml waste plasma bag (anticoagulated with ACD) obtained during the initial plasmapheresis procedure. Plasma was stored at −80°C until use.

Platelet Antibodies

Enzyme-linked immunosorbent assay (ELISA). Antibodies recognizing platelet glycoproteins were detected by using PAKPLUS (GTI, Brookfield, WI). GTI PAKPLUS is a solid-phase ELISA that utilizes affinity purified human leukocyte antigen (HLA) class I antigens and platelet glycoproteins Ib/IX and IV; and glycoproteins IIb/IIIa and Ia/IIa obtained from platelets from donors homozygous for HPA 1a, 1b, 5a and 5b. Assays were performed in duplicate in accordance with the manufacturer's directions.

Flow cytometry. IgG and IgM anti-platelet antibodies were detected in an indirect immunofluorescent flow cytometric assay using target platelets obtained from a single type O volunteer apheresis platelet donor selected for heterozygous expression of relevant platelet glycoproteins. 1.65×10^7 washed platelets resuspended in 0.1% bovine serum albumin/ethylenediaminetetraacetic acid/phosphate-buffered saline were added to patient or normal control plasma with a final plasma to buffer dilution of 1:2. After 40 min incubation at room temperature, platelets were washed and fluorescein isothyocyanate (FITC)-conjugated $F(ab')_2$ fragment goat anti-human IgG (Fc, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and phycoerythrin (PE)-conjugated $F(ab')_2$ fragment donkey anti-human IgM (Fc 5μ, Jackson ImmunoResearch) were added. Samples were analyzed by using a Becton Dickinson FACScan. Single determinations were performed. A positive result was defined as a mean fluorescence intensity ratio of patient plasma to normal plasma greater than 2.0, which is approximately equal to the mean fluorescence intensity value plus four SDs of 24 normal plasma samples.

Endothelial Cell Antibodies

Cell culture. Blood group O human umbilical vein endothelial cells (HUVEC), and group O human dermal microvascular endothelial cells (HMVEC-d, Clonetics, San Diego, CA) were used in third and sixth passage, respectively. Endothelial cells were grown in RPMI 1640 supplemented with 15% heat inactivated horse serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 9 mM HEPES, 645.2 U/ml heparin (all Sigma, St. Louis, MO), and 30 μg/ml endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, MA).

HUVEC activation. Forty-eight hours prior to confluent growth, gamma interferon (Sigma) was added to HUVEC at a final concentration of 200 U/ml and incubated at 37°C for 72 hr. Four hours before harvest, tumor necrosis factor-α, (Sigma) was added at a final concentration of 500 U/ml.

Flow cytometry. Endothelial cells were harvested by incubation with 25 mM ethylenediaminetetraacetic acid/phosphate-buffered saline at room temperature for 12–15 min. Cells were washed and adjusted to a concentration of 1.0×10^6 cells/ml in Hank's balanced salt solution (HBSS, Sigma), with 0.3% bovine serum albumin, 0.1% sodium azide. Cell viability assessed by using trypan blue exclusion was greater than 95%. Patient, or reference plasma from a volunteer type AB blood donor was added in 1:4 dilution to 2.0×10^5 cells. Positive control reagents included human plasma containing antibodies specific for HLA A2, A28, and A9 (ES); human plasma containing an antibody specific for the HPA1 epitope of GP IIIa (KR); or a monoclonal antibody specific for HLA-DR (L243, American Tissue Culture Collection, Rockville, MD). Cells were incubated with plasma or control reagent at 4°C for 1 hr, washed, incubated 30 min at 4°C with FITC-conjugated $F(ab')_2$ fragment mouse anti-human IgG (H + L, Jackson ImmunoResearch Laboratories), then fixed in paraformaldehyde. Antibody stained samples were analyzed by using a Becton-Dickinson FACScan. A positive result was defined as a mean fluorescence intensity ratio of patient to normal plasma of greater than 2.0, which is approximately equal to the mean value plus three SDs of 10 normal plasma samples.

Microlymphocytotoxicity

Samples that demonstrated reactivity in ELISA or flow cytometric assays were analyzed for the presence of HLA antibodies by using the standard National Institutes of Health microlymphocytotoxicity assay augmented with rabbit complement.

TABLE I. Platelet Antibody Specificity

Sample	ELISA	FACS
3	HAL	—
5	HLA, Ia/IIa, IV	—
9	IIb/IIIa	—
13	HLA	IgG
26	HLA	IgG
28	Ia/IIa	IgG
32	HLA	IgG, IgM
37	—	IgM
38	HLA	IgG
39	HLA, Ib	IgG, IgM
40	IIb/IIIa	IgM
43	Ia/IIa	—
44	HLA, IB, IV, Ia/IIa, IIb/IIIa	IgM

RESULTS

Platelet Antibodies

ELISA. Twelve of 48 patient samples (25%) reacted against platelet glycoproteins in ELISA (Table 1). Eight of the twelve reacted with class I HLA antigens. Of these eight, three also contained antibodies against other platelet glycoproteins. Four samples contained only antibodies against platelet glycoproteins including GP IIb/IIIa, Ib/IX, Ia/IIa, and IV.

Flow cytometry. Nine of 48 samples (19%) contained antibodies that bound to allogeneic target platelets (Table I). Eight of nine also contained ELISA-reactive antibodies. Four samples contained IgG only, three contained IgM only, and two contained both IgG and IgM. Platelet reactive immunoglobulins were not detected in four of 13 samples that contained ELISA-reactive antibodies.

Endothelial Cell-Antibodies

HUVEC. Nine of 48 samples (19%) contained antibodies that bound to allogeneic target HUVEC (Fig. 1A). Of the nine, two were also ELISA-reactive against GP IIb/IIIa, and two contained anti-HLA antibodies.

HMVEC-d. To determine if antibodies specific to microvascular endothelial cell antigens are present in TTP patient plasma, we performed antibody-binding studies by using HMVEC-d as target cells. Seven of 48 patient samples (15%) bound to HMVEC-d (Fig. 1B). Of the seven, two contained ELISA-reactive anti-HLA antibodies, one contained both anti-HLA and GP Ib/IX, and one contained anti-GP IIb/IIIa. Three of seven reactive samples also reacted with HUVEC.

Activated HUVEC. In TTP patients, inflammatory conditions might induce expression of new cell surface antigens, creating binding sites for autoantibodies. To determine if TTP patient plasma contains antibodies that bind to activated endothelial cells, HUVEC were stimulated with γ-interferon and tumor necrosis factor-α to induce expression of class II HLA and other antigens.

Six of 48 (13%) patient plasma samples contained antibodies that bound to activated HUVEC (Fig. 1C). Of six, three also contained ELISA reactivity against class I HLA antigens, GP Ib/IX, and GP IIb/IIIa. Four of six reactive samples also reacted with HMVEC-d, and four also reacted with HUVEC.

Microlymphocytotoxicity

Of the twenty samples that were reactive in one or more platelet or endothelial cell assay, eight contained HLA antibodies reactive in microlymphocytotoxicity. Each of these samples also demonstrated reactivity against HLA antibodies in the ELISA.

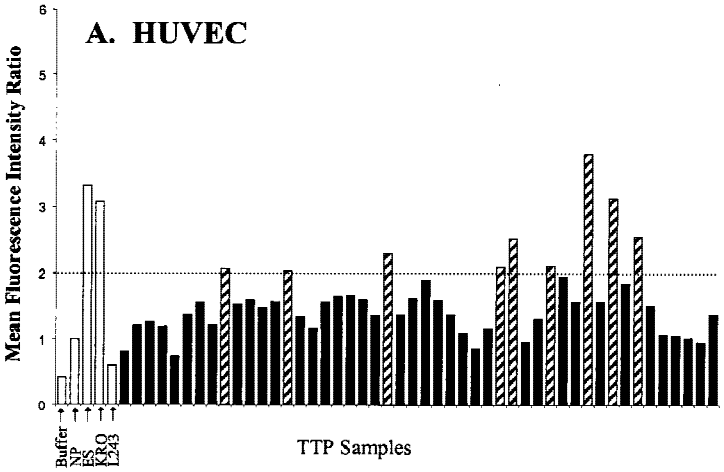
DISCUSSION

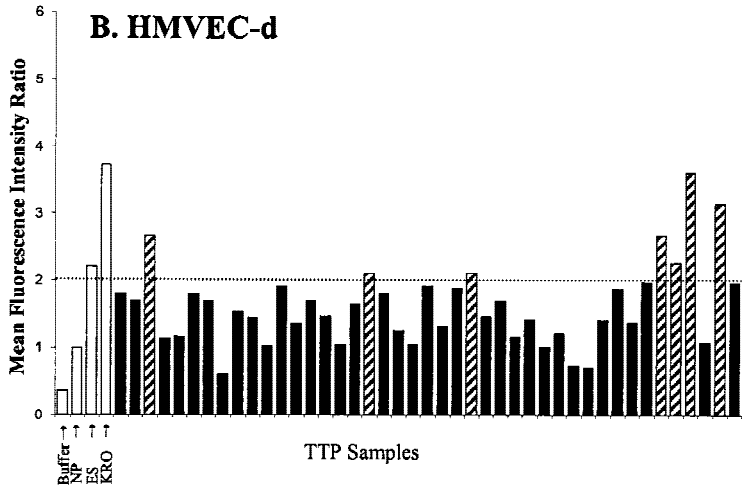
The hypothesis that antibodies against self antigens could mediate vascular injury in TTP is tenable. Autoimmune cytopenias, both idiopathic and drug-dependent, provide models for a possible disorder in which autoantibodies against endothelial cell antigens may occur, resulting in autoimmune endothelial cell injury. Alternatively, inflammatory events in TTP patients could provoke expression of cellular neoantigens or exposure of cryptantigens, creating targets for pre-existing naturally occurring antibodies. To explore the hypothesis that antibody-mediated cellular injury is important in TTP we conducted a systematic search for anti-platelet and anti-endothelial cell antibodies in acute phase TTP plasma samples.

Our results indicate that antibodies that could cause autoimmune injury to platelets or endothelial cells are not prevalent in TTP. Although 42% of samples contained some reactivity, only 25% or fewer were reactive in any one assay. Eight samples (17% of the total samples; 40% of reactive samples) contained HLA antibodies. HLA antibodies could account for all or part of the reactivity observed in these samples, but being alloantibodies, would not be expected to mediate platelet or endothelial cell injury. The observation of 17% HLA antibody positive samples is similar to the prevalence of HLA antibodies in several recent studies of patient and control populations (8% to 15%), indicating that our assays were sufficiently sensitive in detecting HLA alloantibodies [17–19].

Three samples contained anti-GP IIb/IIIa antibodies, two of which reacted with endothelial cells (as might be expected since endothelial cells express GP IIIa). Only 8 of 48 samples (17%) reacted with endothelial cells without an identified antibody specificity. Similarly, only eight samples reacted with platelet antigens other than HLA.

The decades-long search for a common etiologic factor in TTP has yielded a confusing array of data: In 1979 Lian and colleagues first reported the presence of a plate-





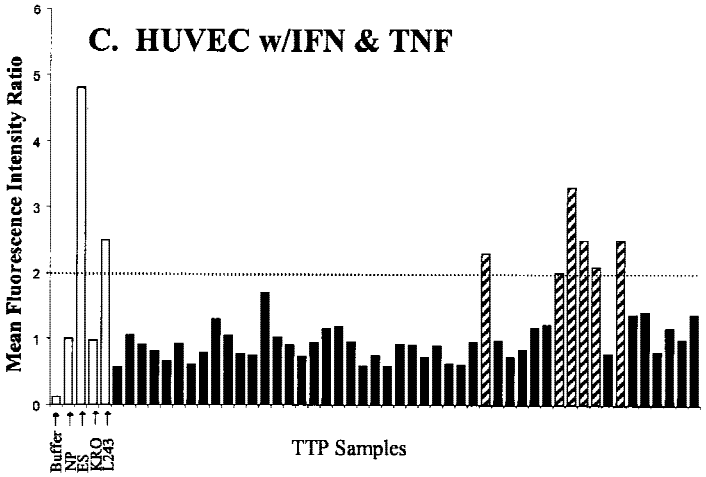


Fig. 1. Flow cytometric analysis of TTP patient plasma antibody binding to endothelial cells. Blood group O HUVEC (A), HMVEC-d (B) or HUVEC stimulated 72 hr with 200 U/ml γ interferon followed by 4 hr stimulation with 500 U/ml tumor necrosis factor-α (C), were incubated with patient or control plasma from a volunteer type AB blood donor (NP), or with human plasmas containing antibodies specific for HLA A2, A28, and A9 (ES); antibody specific for the HPA1 epitope of glycoprotein IIIa (KR); or a monoclonal antibody specific for HLA-DR (L243). Incubation with primary antibody was followed by addition of FITC-conjugated $F(ab^1)^2$ mouse anti-human IgG. Antibody stained samples were analyzed, and ratios of mean fluorescence intensity of patient sample to type AB control plasma (NP) were calculated. Results shown in (A) are mean values of two determinations, (B) and (C) are single determinations. Positive results (hatched bars) are defined as having a ratio greater than 2.0.

let aggregating factor in the plasma of patients with TTP [5]. The implicated factor has been identified as a 37 kDa platelet agglutinating protein (PAP p37) that has been reported to bind to platelet GP IV [20,21]. Other investigators observed similar platelet aggregating activity in TTP plasma or serum [3,22–24]. However, it is unclear whether various investigators identified the same entity. For example, Lian reported a platelet aggregating factor that was inhibited by human IgG [20,21], whereas the aggregating factor reported by Kelton was unaffected by IgG [24,25]. Moreover, platelet aggregating activity and other TTP-associated abnormalities have been detected in plasma from patients with a variety of other diagnoses, suggesting that the phenomena may not be specific to TTP [22,23,26,27]. Therefore, despite a number of published reports, abnormalities in TTP plasma, such as platelet agglutinating factor, have yet to be fully characterized at a molecular level, and the mechanistic roles they may play in the pathophysiology of TTP remain undetermined.

Burns and colleagues reported that sera from three TTP patients contained IgG that bound to cultured endothelial cells [6]. They also observed that acute phase TTP plasmas or sera exerted toxic effects on cultured endothelial cells [6]. Other investigators have reported antiplatelet antibodies in TTP patients with concurrent SLE; antibodies directed against the endothelial cell antigen CD 36 (GP IV); and antibodies against endothelial cell cytosolic and nuclear proteins [10,22,26]. Nakajima and colleagues found that sera from TTP patients inhibited binding of monoclonal antibodies to GP IIb/IIIa, suggesting the presence of blocking anti-platelet or anti-endothelial cell antibodies [28]. In some studies however, antibodies were also found in patients with disorders unrelated to TTP, or in normal plasma [22,26]. Therefore, evidence that antibody-mediated cellular injury is a common etiologic factor in TTP remains controversial.

CONCLUSIONS

We observed that a minority of 48 TTP patients had demonstrable anti-platelet or anti-endothelial cell antibodies in their plasma. Although these studies did not address all possible target antigens, a range of microvascular and macrovascular endothelial cell and platelet antigen substrates were investigated. Therefore, although the pathological significance of the antibodies that were detected is unclear, their infrequent occurrence provides little evidence to support the importance of anti-platelet or anti-endothelial cell antibodies in the pathogenesis of TTP.

ACKNOWLEDGMENTS

We gratefully acknowledge the invaluable services of the Platelet Antibody and Histocompatibility Laboratories of the Blood Center of Southeastern Wisconsin.

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